

## Comparative Pathobiology of Low and High Pathogenicity H7N3 Chilean Avian Influenza Viruses in Chickens

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**SUMMARY.** Chickens were intranasally inoculated with Chilean H7N3 avian influenza (AI) viruses of low pathogenicity (LP) (H7N3/LP), high pathogenicity (HP) (H7N3/HP), and a laboratory derivative (02-AI-15-#9) (H7N3/14D) from the LPAI virus to determine pathobiologic effects. All chickens inoculated with H7N3/HP AI virus became infected and abruptly died 2 or 3 days postinoculation, but a few showed moderate depression before death. The H7N3/HP AI virus produced focal hemorrhages of the comb, petechial hemorrhage at the esophageal–proventricular junction and proventricular mucosa, edema and congestion of the lung, petechiation of the spleen, and generalized decrease in body fat. Histologically, severe necrosis, hemorrhage, and inflammation were primarily identified in lungs and the lymphoid tissues. All tissues sampled from the H7N3/HP AI group were positive for the AI viral antigen, predominantly in endothelium of blood vessels throughout most tissues and less frequently in histiocytes and cellular debris of lymphoid tissues. Even less consistently, cardiac myocytes, hepatocytes, Kupffer cells, glandular epithelial cells, microglial cells, and neurons became infected. These studies suggest the Chilean H7N3/LP AI virus was poorly infectious for chickens and may have been recently introduced from a nongalliform host. By contrast, the H7N3/HP AI virus was highly infectious and lethal for chickens. The H7N3/HP AI virus had a strong tropism for the cardiovascular system, principally vascular endothelium, which is similar to the viral tropism demonstrated previously with other H5 and H7 HPAI viruses. Interestingly, the H7N3/LP AI virus on intravenous inoculation replicated in cardiac myocytes, a feature of HPAI and not LPAI viruses, which further supports the theory that the H7N3/LP AI virus was in transition from LP to HP.

**RESUMEN.** Patobiología comparativa de virus de influenza aviar de baja y alta patogenicidad pertenecientes al serotipo H7N3 aislados a partir de pollos en Chile.

Se inocularon pollos por la vía intranasal con virus de influenza aviar del serotipo H7N3 de baja patogenicidad (H7N3/LP) y virus H7N3 de alta patogenicidad (H7N3/HP) procedentes de Chile, y con una cepa de laboratorio (02-AI-15#9) (H7N3/14D) del virus de influenza aviar de baja patogenicidad del mismo serotipo con el fin de determinar sus niveles de patogenicidad. Todas las aves inoculadas con el virus H7N3 de alta patogenicidad fueron infectadas y murieron en forma abrupta de 2 a 3 días después de la inoculación, algunas presentando síntomas leves de depresión antes de la muerte. Las lesiones producidas por el virus incluyeron hemorragias focales en la cresta, hemorragias petequiales en la zona de unión del esófago con el proventrículo y en la mucosa proventricular, edema y congestión pulmonar, petequias en el bazo y disminución generalizada de los depósitos grasos corporales. El examen histológico reveló necrosis severa, hemorragias e inflamación de los tejidos pulmonares y órganos linfoides. Todas las muestras de tejidos obtenidas del grupo de aves infectadas con el virus H7N3/HP fueron positivas a la presencia de antígenos virales, concentrándose los mismos mayormente en el epitelio de los vasos sanguíneos de los tejidos infectados, y siendo menos evidentes en los histiocitos y detrito celular de los tejidos linfoides. Las células musculares cardíacas, hepatocitos, células de Kupffer, células

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epiteliales glandulares, células de la microglia y neuronas fueron menos consistentes a la presencia de infección por el virus. Estos estudios indican que el virus Chileno de influenza aviar H7N3/LP presenta niveles bajos de patogenicidad en pollos y puede haber sido introducido en las explotaciones de pollos comerciales a partir de hospedadores de especies diferentes a la galliforme. Por otro lado, el virus H7N3/HP presentó altos niveles de infección y mortalidad en pollos. Este virus presenta un tropismo alto por el sistema cardiovascular, principalmente por el endotelio vascular, el cual es similar al tropismo que presentan otros virus de influenza de alta patogenicidad de los serotipos H5 y H7. Se observó replicación viral en las células musculares cardíacas en el grupo de aves inoculadas por vía endovenosa con el virus H7N3/LP, lo cual es característico de los virus de influenza.

Key words: avian influenza, avian influenza virus, high pathogenicity, influenza, immuno-histochemistry, low pathogenicity, pathology

Abbreviations: AAF = amnioallantoic fluid; AGP = agar gel precipitin; AI = avian influenza; BHI = brain-heart infusion; CEF = chicken embryo fibroblast; DPI = days postinoculation; EID<sub>50</sub> = mean embryo infectious dose; H = hemagglutinin, H7N3/14D = 14-day embryo derivative of avian influenza virus A/chicken/Chile/176822/02; H7N3/HP = high pathogenicity avian influenza virus A/chicken/Chile/184240-1/02; H7N3/LP = low pathogenicity avian influenza virus A/chicken/Chile/176822/02; H&E = hematoxylin and eosin; HP = high pathogenicity; IHC = immunohistochemistry; LP = low pathogenicity; N = neuraminidase; NVSL = National Veterinary Services Laboratories; OIE = Office International des Epizooties; PE = plaquing efficiency; SPF = specific-pathogen free

Avian influenza (AI) is caused by a virus that belongs to the family *Orthomyxoviridae*, genus *Influenzavirus A* (25). The virus has surface hemagglutinin (H) and neuraminidase (N) glycoproteins that result in subtype classification into H1 to H15 and N1 to N9. Worldwide, many strains of AI viruses cause various clinical presentations in poultry. AI viruses can be categorized as low pathogenicity (LP) or high pathogenicity (HP) on the basis of the severity of clinical signs and mortality rates produced in experimentally inoculated chickens (25). All HPAI viruses are of the H5 or H7 subtype, but LPAI viruses can be of any of the 15 H subtypes. The clinical signs are extremely variable depending on the virus strain, host species, and presence of secondary factors (23).

HPAI is an extremely infectious and fatal systemic disease of poultry. It produces necrosis, hemorrhage, and inflammation in multiple visceral organs, the brain, and the skin (1,25). However, most AI virus strains are LP and typically cause mild respiratory signs or drops in egg production or, in some birds, no clinical signs. HPAI is a list A disease of the Office International des Epizooties (OIE), whereas LPAI is not a reportable disease under the OIE (25). Some H5 and H7 LPAI virus strains have mutated to HPAI viruses under field conditions, and such a phenomenon has been reproduced in the laboratory (21,25). Epidemiologic surveys have shown that continued circulation of H5 and H7 LPAI in dense poultry populations is associated with the eventual mutation of these strains and the emergence of HPAI viruses.

This scenario has manifested itself four times in the last 20 yr: United States (1983), Mexico (1994–95), Italy (1999–2000), and Chile (2002) (2,5,12,18,22).

In May 2002, an H7N3 LPAI virus was isolated from chickens in the province of San Antonio, Chile, near Santiago (12). In June 2002, additional isolates were determined to be HPAI viruses by the National Veterinary Services Laboratories (NVSL) in Ames, IA (14). On the basis of phylogenetic analysis, the HPAI viruses were determined to have originated from the LPAI virus, but the source was unknown (D. Suarez, unpubl. data).

The purpose of this study was to determine the primary sites of viral replication of both the HPAI and LPAI H7N3 virus isolates of Chile in chickens and to determine their ability to cause disease. In addition, a derivative in 14-day embryonating chicken eggs was generated from the LPAI virus to determine if a HPAI virus could be produced under laboratory conditions and to evaluate its pathobiology for chickens.

## MATERIALS AND METHODS

**Virus propagation.** The H7N3 LPAI virus, A/chicken/Chile/176822/02 (H7N3/LP), and the H7N3 HPAI virus, A/chicken/Chile/184240-1/02 (H7N3/HP), were obtained from the NVSL (courtesy of D. Senne). The working stocks of the viruses were second passage in 10-day-old embryonating chicken eggs via allantoic sac inoculation of the original materials.

Amnioallantoic fluid (AAF) was harvested from the eggs at 30–48 hr postinoculation and diluted 1:300 in brain–heart infusion (BHI) medium to obtain a final inoculum titer of  $10^6$  mean embryo lethal dose per bird. Similarly, sterile AAF diluted 1:300 in BHI was used as the sham inoculum. The derivative, 02-AI-15-#9 (H7N3/14D), was made by inoculation of H7N3/LP stock into 431 14-day-old embryonating chicken eggs. AAF was harvested 48 hr postinoculation from the 361 embryonating chicken eggs that died. The parent virus produced very small plaques at  $10^{-3}$  dilution on chicken embryo fibroblast (CEF) cultures without trypsin. Allantoic fluid from dead embryonating eggs was diluted to  $10^{-4}$  and checked for production of small plaques without trypsin. Those with increased plaquing ability on the preliminary screening tests were further examined for high plaquing efficiency (PE) on CEF cultures in the presence *vs.* absence of trypsin (20). Derivative 9 was selected because it had the highest PE (PE = 0.01%).

**Chickens.** Four-week-old white Plymouth Rock (intravenous pathotyping experiment) and white leghorn (intranasal experiment) chickens of mixed sex were obtained from specific-pathogen-free (SPF) stocks maintained at Southeast Poultry Research Laboratory. All chickens were housed in negative-pressure HEPA ventilated stainless steel isolation cabinets illuminated under continuous light. Feed and water were provided *ad libitum*.

**Experimental design.** The H7N3/LP and H7N3/14D AI viruses were pathotyped by intravenous inoculation in eight 4-wk-old white Plymouth Rock chickens as previously described (26) plus an additional two birds for sampling per virus. At 3 days post-inoculation (DPI), two chickens per group were euthanatized and necropsied, and tissues (as listed in the following paragraph) were fixed in 10% neutral buffered formalin for histopathologic and immunohistochemical evaluation. The H7N3/HP AI virus was not tested in the intravenous pathogenicity test because it was previously shown to be highly pathogenic (14). The chorioallantoic membranes and embryos were examined for viral antigen distribution in 10-day-old SPF embryonating chicken eggs inoculated into the allantoic sac with  $10^6$  mean embryo infectious doses (EID<sub>50</sub>) of the H7N3/HP, H7N3/LP, and H7N3/14D AI viruses, five eggs per virus, and sampled 1–2 DPI. The chorioallantoic membranes and a single transverse midthoracic section of each embryo were fixed in 10% neutral buffered formalin solution and processed for immunohistochemical evaluation as described below.

For the intranasal inoculation experiment, the chickens were separated into four groups: group 1 = sham-inoculated group that served as negative controls; group 2 = H7N3/LP; group 3 = H7N3/14D; and group 4 = H7N3/HP. Group 1 had six birds and was housed in a separate biosafety level 2 facility whereas groups 2–4 each consisted of eight birds and were housed on the

biosafety level 3 agriculture (AG) facility (1). The birds were inoculated intranasally with 0.1 ml of diluted normal AAF (group 1) or 0.1 ml of AAF containing  $10^6$  EID<sub>50</sub> of virus (groups 2–4). The birds were banded and bled on 0 DPI. The chickens were inspected daily for clinical signs. Two birds from groups 1–4 on 1 and 3 DPI and two birds from groups 2–4 on 7 DPI were euthanatized with sodium pentobarbital (100 mg/ml), and tissue samples were collected for histopathology and immunohistochemistry (IHC). The tissues collected included lung, thyroid, thymus, trachea, liver, spleen, heart, pancreas with intestine, intestine (Meckel diverticulum), cecal tonsils, cloacal bursa, kidney, adrenal, gonad, proventriculus, ventriculus, esophagus, comb, eyelid, brain, nasal cavity, eye, and proximal tibiotarsal joint with the physis. On 10 DPI, all survivors (groups 1–4) were bled and then euthanatized, as previously described, and tissue samples were taken. Tissue samples were also taken on days not designated from any animal that died or was euthanatized for being clinically moribund.

**Serology.** Chickens in groups 1–3 were wing bled on days 0, 7, and 10. Because of the 100% mortality by 3 DPI, chickens inoculated with H7N3/HP were bled only on day 0. Blood samples were tested for the presence of influenza virus group-specific antibody by the agar gel precipitin (AGP) test (23).

**Pathology, histopathology, and IHC.** Lesions observed on necropsy were scored as follows: 1 = normal, 2 = mild, 3 = moderate, and 4 = severe. Collected tissue samples were fixed for a minimum of 2 days in a 10% neutral formalin solution after being cut into sections 3 mm or less. In addition, the bone, eye, and nasal cavity were decalcified for 4 days (Regular Cal Immuno Decalcification solution; BBC Biochemical, Stanwood, WA). The tissues were submitted for routine processing to paraffin wax blocks, and 5- $\mu$ m sections were stained with hematoxylin and eosin (H&E) stain for light microscopic examination. The distribution of the influenza A viral nucleoprotein in the individual tissues was detected by IHC staining of duplicate histologic sections with a mouse monoclonal antibody specific for nucleoprotein of type A influenza virus (18).

## RESULTS

**Pathotyping and embryo studies.** In the intravenous pathotyping test, H7N3/LP killed two of eight chickens whereas H7N3/14D killed four of eight inoculated chickens. In two H7N3/LP chickens examined on day 3 after inoculation, lesions consisted of severe widespread nephrosis with lymphocytic interstitial nephritis and edema and associated abundant viral antigen in necrotic tubules. Moderate histiocytic interstitial pneumonia and necrotizing pancreatitis were seen and had associated viral

antigen in pulmonary histiocytes and pancreatic acinar cells, respectively. In addition, viral antigen was seen in surface epithelial cells of cloacal bursa, crypt epithelium of duodenum and jejunum, and scattered cardiac myocytes. The H7N3/14D produced similar lesions and antigen distribution in the cloacal bursa, lung, and kidney.

For the H7N3/LP and H7N3/14D in embryonating eggs, viral antigen was abundant in allantoic epithelium and associated inflammatory cells within necrotic foci. In addition, the H7N3/LP AI virus was visualized in superficial epithelial cells of the skin and feathers and within the epithelial cells of the esophagus. Staining for the H7N3/HP AI virus was intense in allantoic and chorionic epithelium, cardiac myocytes, and all blood vessel endothelium in the chorioallantoic membrane and embryo.

**Intranasal sham-inoculated group.** No morbidity or mortality was observed in the sham-inoculated group. None of the birds had evidence of pathologic changes when observed grossly. On histologic examination, all tissues were normal except the cecal tonsils. Four out of six birds had mild multifocal, heterohistiocytic, cecal tonsillitis with associated bacterial colonies and necrosis. All sampled tissues were negative for AI viral antigen on immunohistochemical staining. The lesions were interpreted to be nonspecific, consistent with bacterial infection of cecal tonsil.

**Intranasal virus-inoculated groups.** *Morbidity and mortality.* One bird inoculated with H7N3/LP virus displayed mild depression on 4, 5, and 6 DPI. In the H7N3/14D group, no clinical sign was evident in any bird. No death occurred in groups H7N3/LP and H7N3/14D. In the H7N3/HP group, one bird exhibited mild depression on 1 DPI whereas two birds were moderately depressed and reluctant to rise on 2 DPI. The H7N3/HP virus resulted in four deaths on 2 DPI and two deaths on 3 DPI, that is, 100% mortality by 3 DPI.

*Gross pathology.* In the H7N3/LP group, mild to

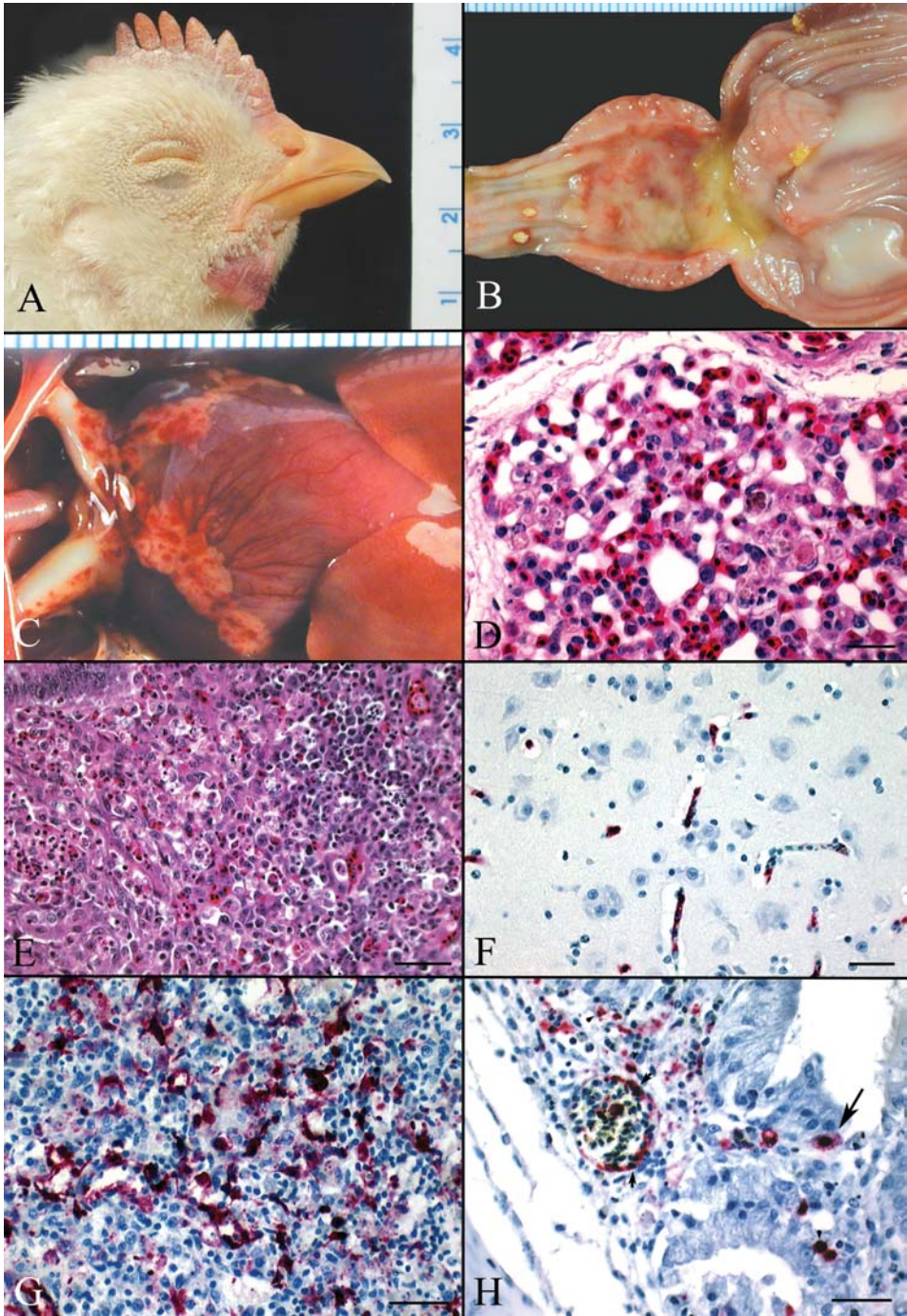
moderately enlarged spleens were seen in two birds on 3 and 7 DPI. In the H7N3/14D group, two of eight birds had mild generalized decrease in body fat and one bird had mild airsacculitis and an enlarged spleen on 1 and 3 DPI, respectively.

For H7N3/HP group, birds euthanized on 1 DPI lacked lesions except for mild focal hemorrhage on the comb of one bird. In the birds that were found dead on 2 DPI, mild focal hemorrhage in the comb was a consistent finding (Fig. 1A). However, one bird had moderate necrosis of the comb without the hemorrhage. Mild edema and congestion of the eyelids and mild-to-moderate generalized decrease in body fat were present in all birds. The lungs had moderate-to-severe edema and mild-to-severe congestion. The trachea and nasal cavity had mild increase in mucus. Moderate-to-severe mucosal hemorrhage at the esophageal–proventricular junction and moderate-to-severe mucosal hemorrhage of the proventricular mucosa were present (Fig. 1B). Single birds had the following: 1) mild hemorrhage in small intestinal submucosal lymphoid aggregates (Peyer patches); 2) mild accumulation of serous fluid at the hilus of the heart and supraorbital edema; 3) petechial hemorrhages in coronary fat pad (Fig. 1C); 4) moderately enlarged cloacal bursa with moderate diffuse petechiation; 5) small amount of free blood emanating from the cloaca; and 6) mild thickening of air sacs. One bird had a moderately enlarged spleen with moderately severe petechiation, whereas two birds had normal sized spleens but with mild petechiation.

On 3 DPI for H7N3/HP group, the two birds had mild-to-moderate decreases in body fat. Mild congestion was present in lungs. Moderate mucosal hemorrhage was seen at the esophageal–proventricular junction and in the proventriculus. Moderate amounts of free blood were emanating from the cloacae. Mild-to-moderate splenomegaly was present and contained a few petechial hemorrhages. A few mild focal hemorrhages were present on the combs.

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Fig. 1. Lesions in chickens inoculated with H7N3/HP. (A) Swollen head, comb, and wattles from edema in chicken that died on 2 DPI. One ruler mark = 1 cm. (B) Hemorrhage in proventricular and ventricular mucosa in chicken that died on 2 DPI. One ruler mark = 1 mm. (C) Petechial hemorrhages in coronary fat in a chicken that died on 2 DPI. One ruler mark = 1 mm. (D) Severe heterophilic to histiocytic interstitial pneumonia with scattered necrosis. Chicken died 3 DPI. H&E. Bar = 25  $\mu$ m. (E) Moderate lymphocyte depletion and cell death with heterophilic inflammation in the cecal tonsil. Chicken died 2 DPI. H&E. Bar = 50  $\mu$ m. (F) AI viral nucleoprotein in capillary endothelial cells of the cerebrum. Chicken died 3 DPI. IHC. Bar = 25  $\mu$ m. (G) AI viral nucleoprotein in histiocytes and endothelial cells of the cecal tonsil. Chicken died 3 DPI. IHC. Bar = 25  $\mu$ m. (H) Common AI viral nucleoprotein in vascular endothelium (small arrows) and inflammatory cells (arrowheads) with occasional AI viral antigen in respiratory epithelium (large arrow). Chicken died 2 DPI. IHC. Bar = 25  $\mu$ m.



The birds had mild supraorbital edema; one bird had conjunctivitis. One bird had mild hemorrhage of the shanks, moderate hemorrhage of the wattle and comb, mild focal necrosis and hemorrhage in the esophagus, and mild supercranial edema.

**Histologic lesions.** Tissues from birds in the H7N3/LP and H7N3/14D groups were normal with the exception of the cecal tonsils and nasal cavity. Six out of eight birds from each group had mild-to-moderate, multifocal, heterophilic cecal tonsillitis with associated bacterial colonies and necrosis. These lesions were similar to those in the sham group and were interpreted as nonspecific bacterial tonsillitis and unrelated to any AI virus inoculum.

In the nasal cavity, four of eight birds from the H7N3/LP group (3,7,10, and 10 DPI) had mild-to-moderate, heterophilic-to-lymphocytic rhinitis with scattered necrosis of associated respiratory epithelium. Two of eight birds from the H7N3/14D group (7 and 10 DPI) had mild, multifocal, heterophilic rhinitis with mild necrosis and epithelial cellular degeneration.

In the H7N3/HP group, birds that died (2–3 DPI) had severe heterophilic-to-mixed cell (heterophils, histiocytes, and lymphocytes) interstitial pneumonia with accompanying mild necrosis in air capillaries and mild-to-severe edema (Fig. 1D). Mild-to-severe, focal-to-diffuse, heterophilic rhinitis and submucosal vasculitis with mild necrosis were common. In addition, moderate, multifocal, heterophilic-to-necrotizing vasculitis was noted in the tracheal submucosa.

All birds in the H7N3/HP group had generalized passive congestion of all visceral organs. Mild, multifocal, myocyte necrosis was common. Lesions in the alimentary tract were largely confined to the lymphoid associated tissues including the esophageal-proventricular junction, cecal tonsils, mucosa of the cecum and jejunum, Meckel diverticulum, and the Peyer patches of the small intestine. Mild-to-moderate, heterophilic-to-histiocytic inflammation was accompanied by severe lymphoid depletion and moderate-to-severe necrosis (Fig. 1E). In the thymus and cloacal bursa, mild-to-severe, diffuse lymphocyte depletion and necrosis were present. Mild, multifocal, heterophilic esophagitis with associated vasculitis was common, but in two birds, severe hemorrhage and necrosis were present in the esophageal mucosa and lamina propria mucous glands. Commonly, birds had mild-to-severe, diffuse, heterophilic-to-heterohistiocytic inflammation of the mucosa at the esophageal-proventricular junction. One bird had severe, diffuse, heterohis-

tiocytic ventriculitis with necrosis. Mild-to-moderate degeneration of individual pancreatic acinar cells and mild diffuse necrosis of the pancreas were common. Mild-to-moderate, diffuse necrosis and mild-to-moderate, histiocytic-to-heterohistiocytic hepatitis were seen consistently. Mild, focal-to-multifocal fibrinoid necrosis was frequent in the livers. Moderate-to-severe lymphocyte depletion and mild-to-moderate necrosis were common in the spleens. Five birds in the H7N3/HP group had mild multifocal gliosis and neuron degeneration in random areas of the brain. The kidneys had mild multifocal areas of necrosis. Mild-to-severe, focal-to-diffuse, heterophilic-to-heterohistiocytic inflammation of the eyelid and comb was common.

**IHC.** Tissues collected from all chickens in H7N3/LP and H7N3/14D groups and two chickens from H7N3/HP collected at 1 DPI were negative for the presence of the influenza viral nucleoprotein. In the birds that died on 2 and 3 DPI, the most consistent detection of antigen was in endothelial cells (Fig. 1F) of capillaries and small venules in multiple visceral organs, brain, and integument (Table 1). The second most consistent staining was in cellular debris and histiocytes (Fig. 1G) within primary and secondary lymphoid tissues (Table 1) and heterophils and monocytes in tissues with inflammatory foci and blood. Less frequently, parenchymal cells of some organs were positive, including cardiac myocytes, Kupffer cells, hepatocytes, pancreatic acinar epithelium, adrenal corticotropic cells, mucous glandular epithelium of esophagus, glandular epithelium of ventriculus and proventriculus, and central nervous system microglial cells and neurons. Nasal cavities of H7N3/HP had most prominent staining of vascular endothelial cells and inflammatory cells (macrophages and heterophils), but epithelial cells were uncommonly stained (Fig. 1H).

**Serology.** AI antibodies were absent in all chickens on the day of inoculation (0 DPI). In the H7N3/LP and H7N3/14D groups at 7 or 10 DPI, one of the four chickens from each group had antibodies against the AI virus.

## DISCUSSION

The H7N3/HP AI virus produced severe clinical disease, lesions, and death similar to those reported for other HPAI viruses (4,7,8,9,10,11,16,17). Most frequent and prominent were gross lesions consisting of hemorrhage at esophageal-proventricular junction and proventricular mucosa, edema and

Table 1. Distribution of the influenza virus in the individual tissues as detected by IHC staining of tissues sampled from the H7N3/HP group.

Tissue	Score <sup>A</sup>			Predominant cell type
	Day 1	Day 2	Day 3	
Nasal cavity	—	+++	+++	BV <sup>B</sup> endothelium > <sup>C</sup> histiocytes and debris in submucosal lymphoid tissue
Trachea	—	+	+	BV endothelium
Lung	—	+++	+++	BV endothelium, histiocytes/monocytes, heterophils
Heart	—	++ to +++	++ to +++	Myocytes and BV endothelium
Bone marrow	—	++	++	Histiocytes > myeloid/erythroid cells
Comb	—	++	++	BV endothelium
Eyelid	—	++ to +++	+++	BV endothelium > inflammatory cells
Esophagus	—	++	++	BV endothelium ≫ mucous gland epithelium
Proventriculus	—	++ to +++	++	BV endothelium > glandular epithelium and histiocytes from lymphoid tissue
Ventriculus	—	+ to ++	++	BV endothelium ≫ glandular epithelium
Duodenum	—	+ to ++	+ to ++	BV endothelium > histiocytes and debris in lymphoid tissue
Jejunum and Meckel diverticulum	—	+ to ++	+ to ++	BV endothelium > histiocytes and debris in lymphoid tissue
Cecal tonsil	—	+ to ++	++	Histiocytes and debris in lymphoid tissue > BV endothelium
Pancreas	—	+ to ++	+ to ++	BV endothelium ≫ acinar epithelium
Liver	—	++ to +++	++	Kupffer cells > sinusoidal endothelium > hepatocytes
Thymus	—	++ to +++	+ to ++	Thymic epithelium > histiocytes > BV endothelium
Cloacal bursa	—	+ to ++	++	BV endothelium > histiocytes
Spleen	—	+++	+++	Histiocytes and debris > BV endothelium
Brain	—	+ to ++	++ to +++	BV endothelium ≫ microglial cells and neurons
Kidney	—	+ to ++	++	Sinus and glomerular endothelium > histiocytes > tubule epithelium
Adrenal	—	++	++	Corticotrophic cells > histiocytes > BV endothelium
Gonad	—	+ to ++	+ to ++	BV endothelium > histiocytes and interstitial cells

<sup>A</sup>— = none; + = mild; ++ = moderate; +++ = diffuse.

<sup>B</sup>BV = blood vessel (capillary and venule primarily but also arterioles).

<sup>C</sup>> = frequency of the first is greater than that of the latter; ≫ = frequency of the first is much greater than that of the latter.

congestion of lungs, and edema and hemorrhage of comb and wattles. These changes suggest one or more pathologic mechanisms focusing on alterations to the cardiovascular system. In addition, the histopathologic lesions of severe pulmonary edema in the air capillaries and extensive vasculitis, and widespread demonstration of AI viral antigen in blood vessel and sinusoidal endothelial cells, support

a prominent vascular endothelial cell tropism for virus replication and generalized circulatory collapse as the major mechanisms involved with production of disease and sudden death. Other HPAI viruses, such as 1997 Hong Kong H5N1 (11,16,17), 1994 Australian H7N3 (7), 1999–2000 Italian H7N1 (4), and 1991 England H5N1 avian influenza viruses (8,9), had vascular endothelial tropism with

cell death and subsequent production of edema and necrosis. Furthermore, the 9-day chicken embryos in the current study had prominent vascular endothelial cell and cardiac myocyte replication, as has been reported for the Italian H7N1 HPAI viruses (3), which further supports a prominent cardiovascular mechanism for injury and death. In the current study, dissemination of the virus via the circulatory system may have been via blood leukocytes as supported by demonstration of AI viral antigens in monocytes and heterophils.

Secondarily, depletion and cell death in lymphocytes and demonstration of AI viral antigen were noted in primary and secondary lymphoid tissues. The AI viral nucleoprotein was seen in histiocytes and cellular debris but not in lymphocytes. Previous studies have disagreed on the role of lymphoid tissues in HPAI pathogenesis. *In vitro* infection of chicken lymphocytes and macrophages has been demonstrated with HPAI virus isolate A/turkey/Ontario/66 (H5N9) but not with HPAI virus isolates A/chicken/Pennsylvania/1370/83 (H5N2) and A/tern/South Africa/61 (H5N3) (27). However, other authors have demonstrated *in vivo* lymphocyte depletion and cell death in primary and secondary lymphoid tissues without demonstration of AI viral proteins in lymphocytes (7,9,11). The mechanism by which such lymphocyte cell death occurs is unclear, but, on the basis of biochemical and morphological studies, apoptosis or necrosis or both may be involved (11,13,27). Most favor release of cytokines from AI virus-infected macrophages or other cells and such cytokines inducing apoptosis in lymphocytes (13,27). The morphologic lesions in the current study suggest apoptosis as the mechanism of cell death for lymphocytes, but in parenchymal cells of various types, necrosis was a more consistent finding. In addition, demonstration of AI viral nucleoprotein in histiocytes, Kupffer cells, and other related phagocytic cells suggests a prominent role for cells of the monocyte-phagocyte system in virus replication and dissemination.

In the current study, after the vascular endothelial replication and dissemination, less consistent AI viral replication was noted in various parenchymal cells including cardiac muscle, hepatocytes, pancreatic acinar cells, adrenal corticotrophic cells, glandular epithelium of the intestinal tract, and microglial cells and neurons of the central nervous system. Previous studies with other HPAI viruses have shown replication and damage to parenchymal cells of various organs (5,8,9,11,16,17). Such

replication was most frequently evident in birds that survived the peracute stage of the disease (24–48 hr, that is, blood vessel endothelial cell replication, which allowed sufficient time for the HPAI virus to be disseminated to parenchymal cells of various organs and undergo several replication cycles before the death of the bird. This parenchymal cell tropism was particularly a prominent feature in experimental studies with A/chicken/Pennsylvania/1370/83 (H5N2) and 1999 Hong Kong H5N1 AI viruses (5,9). Furthermore, chickens inoculated via the intravenous route tended to die earlier than those inoculated via the intranasal or intratracheal route (5). The intravenously inoculated birds died during the peracute stage (24–48 hr) and had primarily endothelial cell replication and damage with minor involvement of parenchymal cells. After inoculation by the intranasal or intratracheal route, vascular endothelial cell replication was less prominent and replication in parenchymal cells was more prominent.

In the current studies, intranasal inoculation with H7N3/LP and H7N3/14D AI viruses resulted in mild lesions in the nasal cavity of a few chickens and no death. Such respiratory tropism has been reported previously with intranasal inoculation of LPAI viruses in chickens (9,20). However, the H7N3/LP and H7N3/14D AI viruses in the intravenous pathogenicity test produced deaths in two of eight and four of eight chickens, respectively. Previously, LPAI viruses have been shown to cause no mortality or occasionally death in one of eight inoculated chickens but rarely death in two of eight (6,15). However, more interesting in the current study was demonstration of viral antigen in cardiac myocytes from chickens in the H7N3/LP AI virus group. Myocardial localization has been common with HPAI virus infections but rare in LPAI virus infections (4,19,21,22,24), suggesting a unique pathobiologic effect of the H7N3/LP AI virus infection and a possible indication that this LPAI virus was in an early stage of transition from LP to HP.

Previous studies in our laboratory by the 14-day embryonating chicken egg system have produced 47% and 15% emergence rates of HPAI virus derivatives from H5 and H7 LPAI viruses tested, respectively (20). In the current study, lack of mortality in the intranasal H7N3/14D group suggests the 14-day derivative was biologically a LPAI virus when given by a natural route of inoculation (i.e., intranasal) and had an upper respiratory tract tropism. However, failure to derive

a HPAI virus does not disprove the H7N3/LP virus from being the parent virus that gave rise to the H7N3/HP virus in the field. Phylogenetic comparison of the hemagglutinin gene supports H7N3/LP giving rise to the H7N3/HP virus through insertion of 30 additional bases from AI viral nucleoprotein that coded for 10 amino acids (D. Suarez, pers. comm.).

On the basis of the poor serologic response and infrequent production of lesions, the H7N3/LP and H7N3/14D viruses did not consistently replicate in chickens or produce a consistently measurable serologic response. In previous studies, intranasal inoculation with  $10^6$  EID<sub>50</sub> of two LPAI viruses resulted in 63% and 100% seroconversion at 5 DPI and 100% by 10 DPI (22). In the current experiment, only 25% of the birds were serologically positive at 7 DPI even though they received the high inoculation dose of  $10^6$  EID<sub>50</sub>. In a previous study,  $10^{2.8}$  EID<sub>50</sub> of AI virus isolate A/turkey/Virginia/158512/02 (H7N2) resulted in a 50% infection rate in white leghorn chickens whereas 100% infection rate was obtained with  $10^4$  EID<sub>50</sub> of the AI virus (D. E. Swayne, unpubl. data). By contrast, two feral waterfowl-origin AI viruses required  $10^{7.6}$  and  $10^{8.3}$  EID<sub>50</sub> to infect 50% of intranasally inoculated white leghorn chickens, but 100% infection rates were not achievable with higher virus doses. In another study, 25% of white Plymouth Rock chickens were AGP serologic positive after intranasal inoculation with an H7N1 LPAI virus isolated from a rhea (*Rhea americana*). When  $10^{6.9}$  EID<sub>50</sub> of this virus was given intranasally, a 50% infection rate was produced in white Plymouth Rock chickens. These data suggest the H7N3/LP and H7N3/14D AI viruses were poorly adapted to gallinaceous birds (i.e., chickens, turkey, and quail) and the H7N3/LP was recently introduced from another avian species, possibly a feral waterfowl or another nongallinaceous bird species.

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